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Purification and Characterization of the Sin Nombre Virus Nucleocapsid Protein Expressed in *Escherichia coli*

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Sin Nombre virus is a member of the Hantavirus genus, family *Bunyaviridae*, and is an etiologic agent of hantavirus pulmonary syndrome. The hantavirus nucleocapsid (N) protein plays an important role in the encapsidation and assembly of the viral negative-sense genomic RNA. The Sin Nombre N protein was expressed as a C-terminal hexahistidine fusion in *Escherichia coli* and initially purified by nickel-affinity chromatography. We developed methods to extract the soluble fraction and to solubilize the remainder of the N protein using denaturants. Maximal expression of protein from native purification was observed after a 1.5-h induction with IPTG (2.4 mg/L). The zwitterionic detergent Chaps did not enhance the yield of native purifications, but increased the yield of protein obtained from insoluble purifications. Both soluble and insoluble materials, purified by nickel-affinity chromatography, were also subjected to Hi Trap SP Sepharose fast-flow (FF) chromatography. Both soluble and insoluble proteins had a similar A_{280} profile on the Sepharose FF column, and both suggested the presence of a nucleic acid contaminant. The apparent dissociation constant of the N protein, purified by nickel-affinity and SP Sepharose FF chromatography, and the 5′ end of the viral S-segment genome were measured using a filter binding assay. The N protein–vRNA complex had an apparent dissociation constant of 140 nM.

Hantaviruses cause two illnesses in humans, hemorrhagic fever with renal syndrome (HFRS) and hantavirus cardiopulmonary syndrome (HCPS) (1). Hantaan virus (HTNV) and Sin Nombre virus (SNV) are the major causative agents of HFRS and HCPS, respectively. Hantaviruses have a negative-sense, single-stranded RNA genome that consists of three segments, S, M, and L (2, 3). Transcription of each vRNA by the viral-encoded RNA-dependent RNA polymerase (RdRp) yields a complementary RNA (cRNA), which in turn is used as a template to generate vRNA by the RdRp. In contrast to the hantaviral mRNAs, which do not associate with the viral nucleocapsid protein (N) (4), the vRNA and cRNA replicative templates are encapsidated by N. The interactions of the hantaviral RNAs and N are not well defined. Gott *et al.* showed the presence of a nonspecific RNA binding domain in the C-terminus of the HTNV N protein (5). In another study, the HTNV N bound preferentially to the viral genome rather than to nonviral RNA (6). More extensive biochemical studies of RNA–protein or protein–protein interactions will require sufficient quantities of highly purified, soluble, monodispersed N protein that are free of contaminating ribonucleases, nucleic acids, and RNA binding proteins. Toward this goal, we have investigated methods to recover hantaviral N from a bacterial expression system in a form suitable for biochemical analysis.

Our previous purification strategy, as well as that of others, relied on using denaturing methods to recover soluble expressed N from bacteria (5, 6). Because renaturing does not always result in native protein, large losses in functional N are common. Comparing yields of HTNV and SNV N after denaturing and renaturing

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shows that the SNV N protein is more readily recovered and probably is more stable (6). No obvious reason for the difference in ease of recovery of soluble SNV N versus HTNV N can be deduced from examining the amino acid sequences of the two proteins. The most notable difference is the substitution of seven Gly residues in the HTNV N protein with Asn, Ala, Ser, or Asp in the SNV N protein. All of these substituted amino acids are more hydrophilic than Gly (7), and if they are located on the surface of the protein, they could facilitate the interaction of the protein with an aqueous environment. Insight into the molecular basis for the differences awaits a three-dimensional structure that could provide information on the solvent accessibility of these residues.

Herein, we report studies leading to the development of a rapid soluble extraction protocol and chromatography method for recovery and purification of the SNV N protein expressed in *Escherichia coli*. We further test this method for suitability for preparing N proteins of three other hantaviruses.

MATERIALS AND METHODS

Reagents and Expression Vectors

Restriction enzymes and Vent polymerase were purchased from New England Biolabs (Beverly, MA). T4 DNA ligase and kinase were purchased from Gibco BRL (Grand Island, NY). All chemicals were purchased from Sigma (St. Louis, MO). The plasmid expression vectors pSEO-N pET-1 and pPUU-N pET-1 (gift from Brian Hjelle, M.D., University of New Mexico) contain the open reading frames (ORF) for the Seoul virus 80/39 (SEOV) and Puumala virus P360 (PUUV) N proteins (8). HTNV N protein was expressed from pHTNV-N as described previously (6).

Construction of the SNV N Protein Expression System

SNV, strain CC107, S-ORF was amplified by PCR from SNVS/pCRII (9) with two DNA oligonucleotide primers, SNV S-Nhe, 5′-TCACTGGATTCCATATGGCTAGCACCTCAAAGAATGC, and SNV S-Xho, 5′-TCACTGGATTCTTACTCGAGAAGCTTAAGTGGTTCCTGGTTAGAAATTTC. Primers were synthesized with an ABI 394 DNA/RNA synthesizer. The SNV S-5′ ORF contained a *Nhe*I restriction enzyme digestion site at the 5′ end (underlined) and the SNV-3′ ORF contained a *Xho*I digestion site at the 5′ end (underlined). The PCR product and the pET21b expression vector were digested with *Nhe*I and *Xho*I (New England Biolabs). The products were separated by agarose electrophoresis and purified with the QIAquick gel extraction kit (Qiagen) according to the manufacturer's protocol. The SNVS-ORF and pET21b were ligated with T4 DNA ligase (Gibco BRL). Clones of SNVN/pET21b were selected by restriction enzyme mapping and confirmed through DNA sequencing using the LiCOR 4200 IR^2 automated sequencer. SNVN/pET21b was transformed into competent *E. coli* BL21(DE3) cells (Novagen) for expression studies.

Expression and Purification of SNV N Protein

In general, *E. coli* BL21(DE3) cells harboring the SNVN/pET21b and other hantavirus N proteins were grown overnight in 200 ml of Luria–Bertani (LB) medium containing 200 μg/ml ampicillin. After 16–19 h, cells were diluted 1:20 in LB medium containing 200 μg/ml ampicillin and grown for 1 h at 30°C at which time isopropylthiogalactoside (IPTG) was added to a final concentration of 0.6–0.8 mM to induce expression of the protein. The methods that follow are based on a harvest of an 800-ml bacterial culture grown in a 2-liter flask. Soluble and insoluble extractions were performed and subjected to both metal chelate-affinity chromatography and ion-exchange chromatography. In general, fractions were collected and 20 μl of each fraction was examined for SNV N protein by SDS–PAGE or separation by 4–12% NuPAGE Novex Bis-Tris Gels (Invitrogen) and Western blot analysis. Western blots were performed using polyclonal rabbit sera to SNV N (diluted 1:4000) or mouse sera to polyhistidine (diluted 1:2000) (Sigma; monoclonal clone His-1). The secondary antibodies were alkaline phosphatase-conjugated anti-mouse or anti-rabbit IgG (diluted 1:1000) (Promega), which were developed using Western Blue Reagent (Promega). In addition, protein concentrations were measured using the Bradford method (10) with Bio-Rad Micro-Assay reagents as recommended.

Purification Using Nickel Nitriloacetate Agarose under Native Conditions

After induction with IPTG for 1.5–5 h, 800 ml of the culture were harvested and resuspended in 50 ml of ice-cold solubilization buffer [50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, protease cocktail inhibitor (Boehringer Mannheim), and 0.50 mg lysozyme with or without Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate)]. The suspension was Dounce homogenized for 30 min on ice. Following lysis, the material was sonicated five or six times on ice, with a cycle consisting of 1 min on and 2 min off, in a Branson Sonifier until the material clarified. Soluble and insoluble materials were separated by centrifugation at 30,000*g* for 1 h. Insoluble material was saved and placed at −80°C for subsequent extraction. To the soluble fraction, 1 ml of a 50% suspension of nickel nitriloacetate agarose (Qiagen, Chatsworth, CA) was added per liter of starting material and stirred at 4°C for 2 h

to overnight. The material was added to a column and washed with 10 column volumes of wash buffer [50–100 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole, 20 mM β-mercaptoethanol (BME)]. The N protein was eluted from the resin with elution buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 250 mM imidazole, 20 mM β-mercaptoethanol). Fractions were either stored at −80°C or directly placed into 500 ml of dialysis buffer [40 M Hepes, pH 8.0, 200 mM NaCl, 0.1 mM dithiothreitol (DTT)] for 2–4 h with two changes of buffer.

Purification Using Nickel Nitriloacetate Agarose under Nonnative Conditions

Insoluble material was resuspended on ice in 50 ml denaturing buffer (DB; 50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM BME, 8 M urea, with or without 10 mM Chaps). The suspension was Dounce homogenized and sonicated as described above. The extract was gently shaken at room temperature for an additional 30 min and then centrifuged at 30,000*g* for 1 h. The supernatant was applied to a 1-ml column preequilibrated with DB, pH 8.0. The column was washed with 10 column volumes of DB, pH 8.0; DB, pH 6.3; and DB, pH 5.9. The N protein was eluted with 10 column volumes of DB, pH 4.5. One-milliliter fractions were collected and 10 μl of each fraction was examined for SNV N protein by SDS–PAGE and Western blot analysis. Fractions containing N were dialyzed over a 5-day period into a final buffer with 20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 500 mM NaCl, 5% glycerol, with urea concentrations decreasing daily (day 1—4.0 M, day 2—2.0 M, day 3—1.0 M, day 4—0.5 M, day 5—0 M).

Purification of SNV N Protein under Native Conditions with SP Sepharose Chromatography

Material isolated from soluble or insoluble/refolded fractions was subjected to an overnight dialysis into 50 mM Mes, pH 6.2, 200 mM NaCl. Fifty milliliters of the dialyzed material was then bound to a 1-ml Pharmacia Hi Trap SP Sepharose fast-flow column preequilibrated with 50 mM Mes, pH 6.2, 200 mM NaCl at a flow rate of 0.5 min/ml. A 10-ml gradient from 200 mM to 1 M NaCl was run by the FPLC at a flow rate of 1.0 ml/min. One-milliliter fractions were collected and 20 μl of each fraction was examined for SNV N protein by SDS–PAGE and Western blot analysis as described above.

Filter Binding Assay of SNV N Protein

The filter binding assays were as described previously (6) using synthetic RNA and purified N protein. The oligoribonucleotide corresponding to the 5′ end of the SNV S-segment vRNA (5′-UAgUAgUAgACACCUUgAAAAgCAAUCAAgAAUUUACUU-3′) was synthesized and HPLC purified by Integrated DNA Technologies, Inc. (Coralville, IA). Synthesis was performed on a 1 μM scale. The synthetic RNA was labeled at the 5′ terminus with [(γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and purified on Quick Spin columns (Roche). SNV N protein was serially diluted in binding buffer (40 mM Hepes, pH 7.4, 100 mM NaCl, and 5% glycerol) to give a final concentration range of 5.6×10^{-9} to 5.6×10^{-6} M. Apparent dissociation constants (K_d) were calculated by fitting a nonlinear binding curve to the empirical data using the Origin program (MicroCal). The apparent K_d corresponds to the concentration of N protein required to obtain half-saturation; assuming the complex obeys a simple binding bimolecular equilibrium. We assumed the plateau in the percentage binding of the RNA represents complete binding of the RNA, to allow the calculation at half-saturation.

Dynamic Light Scattering of SNV N Protein

SNV N protein was examined by a DynaPro LSR (Protein Solutions, Inc.) to determine its molecular weight. Measurements were made in the buffers in which the proteins were eluted from the column.

RESULTS AND DISCUSSION

Optimization of Soluble Extraction and Purification Conditions for the SNV N Protein

Small-scale (800 ml) bacterial cultures were used to qualitatively define the optimal extraction and nickel-affinity purification conditions for the SNV N protein. Three extraction/running buffers were examined: buffer 1—100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 30 mM imidazole; buffer 2—100 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 5% glycerol; buffer 3—100 mM sodium phosphate, pH 8.0, 1 M NaCl, 10 mM imidazole, 5% glycerol. Buffer 1 resulted in the greatest yield of soluble SNV N protein. Comparison of buffer 1 with an identical buffer with 10 mM imidazole showed that the higher concentration of imidazole did not reduce background binding of nonspecific proteins; therefore, the imidazole was lowered to 10 mM in subsequent experiments.

Purification of the SNV N Protein Following IPTG Induction

Induction periods of 1.5 or 5.0 h were tested for influence on SNV N protein yield from 3200-ml cultures of bacteria. N protein was extracted and purified by nickel-affinity chromatography under native conditions as described under Materials and Methods. Fractions

from each of the purifications were examined by Coomassie blue staining (Figs. 1A and 1B) as well as by Western blot analysis (data not shown). The N protein had an apparent molecular weight slightly greater than its predicted molecular weight of 49 kDa (11). The total yield of protein in fractions 1–4 from the 1.5-h induction was 7.6 mg, or 2.4 mg/L, while the yield from the 5-h induction was 3.3 mg, or 1.0 mg/L (Table 1). Thus approximately twice as much total protein was recovered following the shorter induction period.

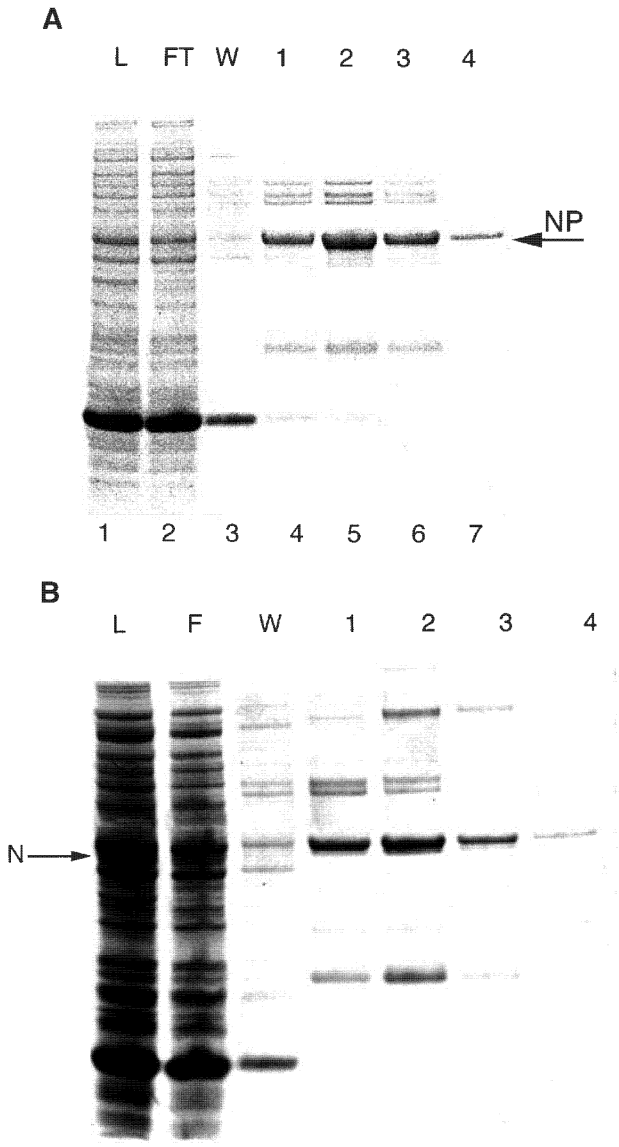

FIG. 1. Influence of IPTG induction time on SNV N yield. 3.2-L cultures of bacteria expressing SNV N were extracted at 1.5 (A) or 5.0 h (B) after adding IPTG. Proteins were extracted with Chaps and purified by nickel-affinity chromatography and analyzed by electrophoresis on 4–12% Bis-Tris gels and Coomassie blue staining. L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. An arrow points to the N protein.

TABLE 1

Summary of Ni-NTA Purification Yields for Various N Proteins

Hantaviral N protein	Induction (h)	Yield (mg/L)	Chaps added	Extraction method[a]
SNV	1.5	2.4	No	N
	1.5	2.4	Yes	N
	5.0	1.0	No	N
	1.5	0.09	Yes	D
	1.5	0.07	No	D
SEOV	1.5	0.45	Yes	N
PUUV	1.5	1.3	Yes	N
HTNV	1.5	ND	Yes	N

Note. ND, not determined.
[a] N, native; D, denaturing.

Expression and Purification of the SNV N Protein in the Absence of Chaps

The zwitterionic detergent, Chaps, has been frequently used to enhance the solubility of proteins recovered during both native and denaturing extraction protocols (12). Thus, our initial extraction and running buffers included this detergent. To determine if Chaps enhanced the solubility and yield of the SNV N protein, we extracted bacterial cells and performed nickel chromatography using the same native conditions as described above for the time-course study, but without the addition of Chaps. Fractions from each of the purifications were examined by Coomassie blue staining (Fig. 2) as well as by Western blot analysis (data not shown). The total yield of protein obtained in fractions 1 through 4 was 7.7 mg (2.4 mg/L). This yield was similar to that observed when Chaps was included in the extraction buffer (Table 1), although inclusion of Chaps resulted in a higher proportion of the total protein in fraction 2 of the elutant (Figs. 1A and 1B). In contrast to these results, inclusion of 10 mM Chaps in the denaturing protocol did enhance SNV N recovery (Table 1 and Figs. 3A and 3B). Overall, the native extraction protocol, irrespective of the inclusion of Chaps, yielded a greater amount of N protein than the denaturing methods; however, the denaturing protocol (Figs. 3A and 3B) yielded a much more homogeneous and pure preparation of N than the native extraction (Figs. 1 and 2).

Expression and Purification of the Other Hantavirus N Proteins Using Methods Developed for the SNV N

In previous studies, we were unable to purify HTNV N protein expressed in *E. coli* using native conditions, although we could recover some protein (approximately 1.5 mg per liter of culture) from the insoluble fraction using denaturing conditions (6, 12).

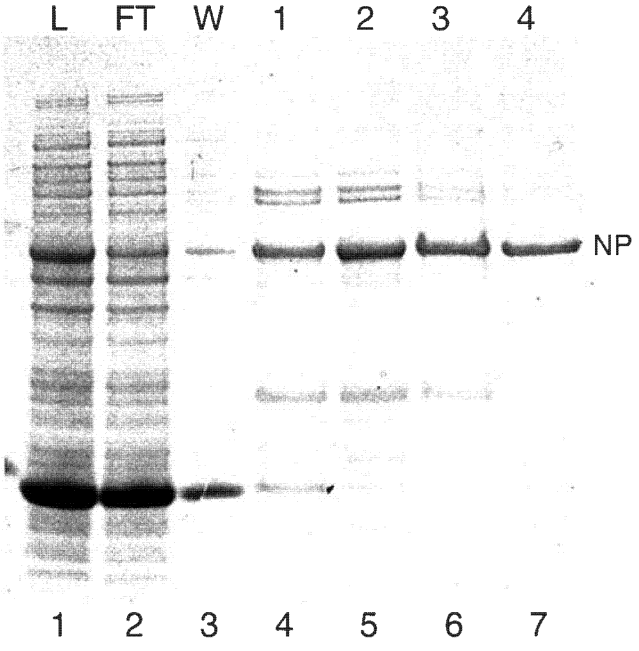

FIG. 2. Native purification of the SNV N protein by Ni-NTA chromatography without Chaps. A representative purification from cellular material extracted without Chaps is shown. L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

To determine if the methods that we devised for extraction and purification of soluble SNV N would be applicable to other hantavirus N proteins expressed in *E. coli*, we tested them for recovery of expressed N of three hantaviruses that cause hemorrhagic fever with renal syndrome: HTNV, PUUV, and SEOV. Proteins were extracted with the nondenaturing buffer containing Chaps and isolated by nickel-affinity chromatography. Eluted fractions were separated by electrophoresis on gradient gels and visualized by Coomassie blue staining (Figs. 4A–4C). The total yield of PUUV or SEOV V N proteins from 1 liter of culture was 1.3 or 0.45 mg/L, respectively (Table 1). The yield of HTNV protein was difficult to compare to the other N proteins because of higher background; nevertheless, the amount visualized by Coomassie blue staining was much greater than we previously observed using denaturing and refolding methods.

Purification of the SNV N Protein by SP Sepharose Fast-Flow Chromatography

To further refine our purification protocol for SNV N, we subjected material recovered by nickel-affinity chromatography from both the soluble and the insoluble refolded fractions to chromatography on a 1-ml Pharmacia SP Sepharose FPLC column with a gradient of 200 mM to 1 M NaCl (Fig. 5A). Before being loaded onto the FPLC column, the nickel-affinity column fractions were pooled and dialyzed overnight against 50 mM Mes, pH 6.2, 200 mM NaCl. This dialysis step enhanced the binding of the protein on the SP Sepharose matrix. From both the native and the refolded protein preparations, two peaks were evident in the FPLC column A_{280} profile. Figures 5A and 5B show the profiles for the N protein recovered following refolding. Examination of the proteins in those peaks by SDS–PAGE revealed little N protein in the first peak (Fig. 5B, lanes 1 and 2). The A_{260}:A_{280} ratio of this peak was 1.7, which strongly suggests the presence of nucleic acids in these fractions. The second peak, which eluted at 9 min in 660 mM NaCl, had most of the N protein (in both refolded and soluble preparations) (Fig. 5B, lanes 3 and 4). These data suggest that the refolded material has properties similar to the native material. At present, we estimate that the isolated N protein has 85% or greater homogeneity. A summary of the yields from the NTA and SP Sepharose FF is presented in Table 2.

Light-scattering analysis of N protein showed that the N proteins isolated from the nickel-affinity resin and the SP Sepharose were 96 and 87% monodispersed, respectively (data not shown). As reflected in the results

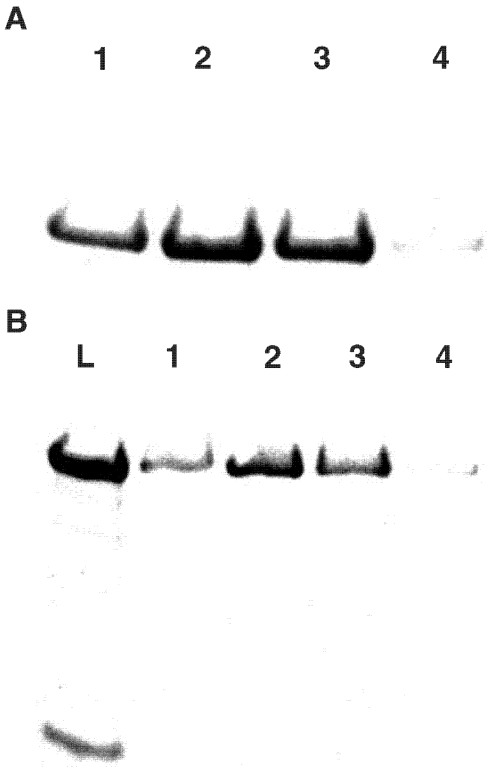

FIG. 3. Denaturing purification of the SNV N protein in the presence and absence of Chaps by NTA chromatography. Purifications are shown from insoluble cellular material extracted with Chaps (A) and without Chaps (B). L, column load; 1–4, fractions eluted from column. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

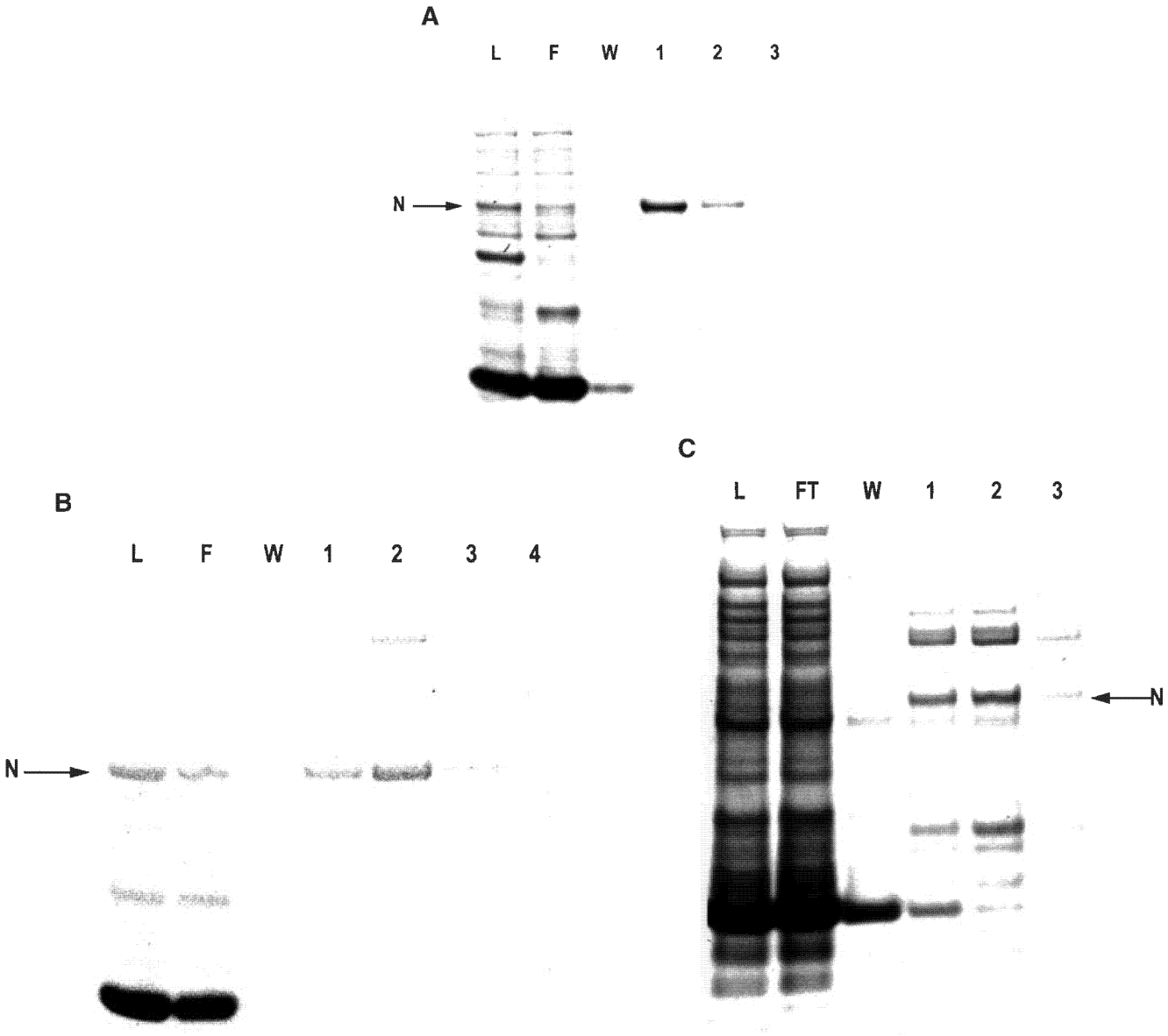

FIG. 4. Purification of PUUV, SEOV, and HTNV N protein by NTA chromatography. Purifications are shown for cellular material extracted with Chaps for PUUV (A) and SEOV (B) and HTNV N protein (C). L, column load; F, column flowthrough; W, column wash; 1–4, fractions eluted from column. An arrow points to the N protein. These samples were subjected to separation by 4–12% NuPAGE Novex Bis-Tris gels.

stated above, the molecular mass of the N protein isolated by the nickel-affinity resin, 87 kDa, was greater than the predicted molecular mass, 49 kDa, and supports the presence of a nucleic acid contaminant. Light scattering showed that the N protein isolated by SP Sepharose chromatography (Fig. 5B, lanes 3 and 4) had a molecular weight of 54 kDa.

RNA Binding Activity of the SNV N Protein

To determine if the extraction and purification protocol that we devised resulted in SNV N protein suitable for biochemical studies, we tested the purified protein in an RNA binding assay. Based on earlier findings suggesting that the terminal nucleotides of the hantaviral S-segment are involved in encapsidation and nucleocapsid assembly (13), we prepared a 39-base synthetic oligoribonucleotide for the binding assay. This oligonucleotide, designated SNV-vRNA 1-39, corresponds to the 5′ end of the nascent S-segment vRNA. Filter binding experiments performed with increasing concentrations of purified SNV N protein and a constant amount of SNV vRNA were used to generate a binding isotherm. The apparent dissociation constant (K_d), calculated as half-maximum binding, was approximately 140 ± 30

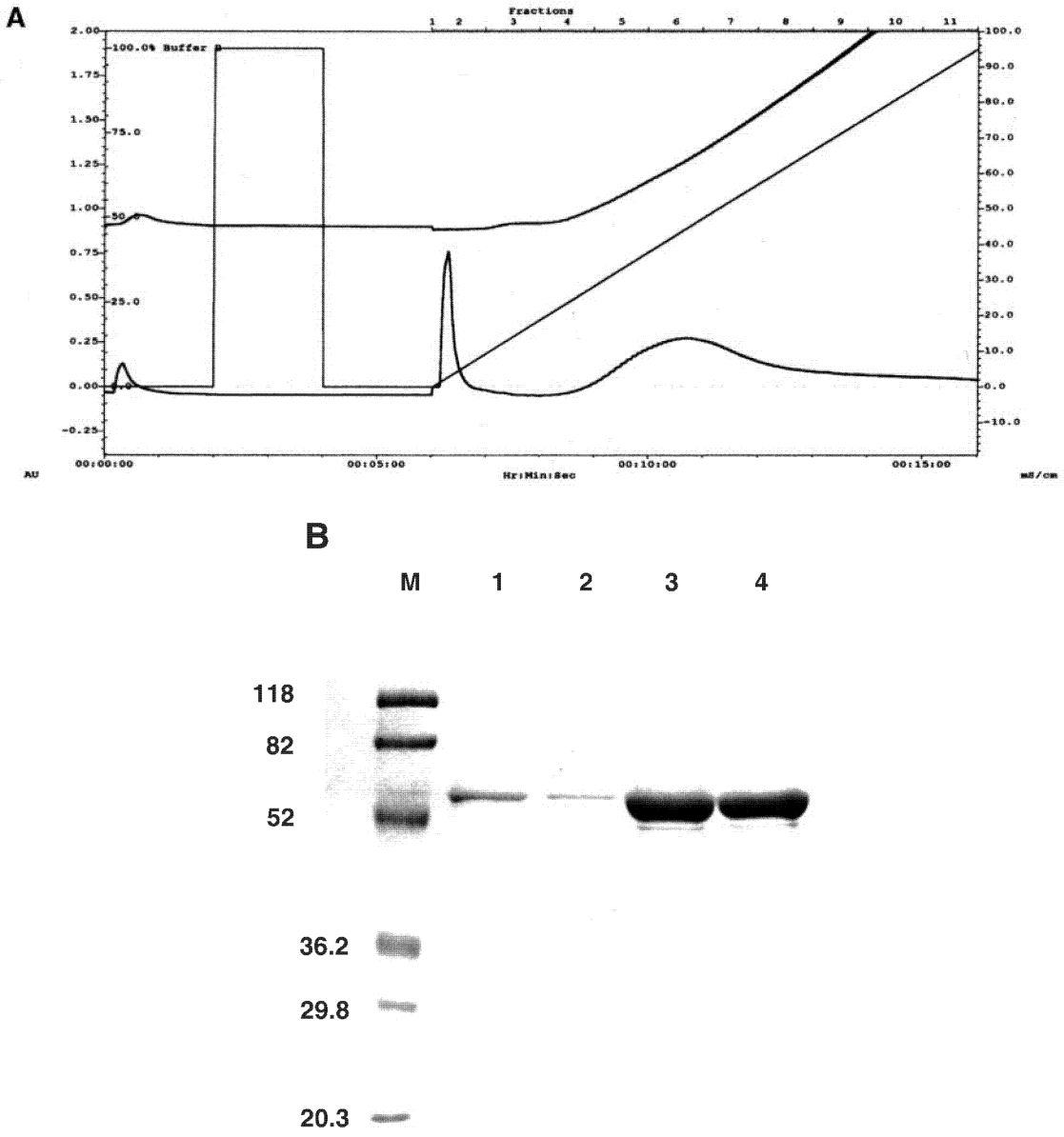

FIG. 5. SP Sepharose chromatography of SNV N protein. (A) 100 ml of refolded N protein was loaded onto a 1-ml Pharmacia SP Sepharose column. The column profile shown was run from 200 mM to 1 M NaCl. (B) Fractions from the two major peaks shown in the chromatogram in (A) are presented: Peak 1, fractions 1 and 2 (lanes 1 and 2); peak 2, fractions 5 and 6 (lanes 3 and 4). These samples were subjected to separation by 12% SDS–PAGE.

nM. This interaction shows a similar binding affinity reported for the HTNV N-protein vRNA (1-39) complex (132 ± 9) (13). We suggest that this specific oligoribonucleotide substrate competed for N protein binding, thereby displacing any remaining nucleic acid contaminant. These data are further supported by recent competition experiments with the HNTV N protein (13). These data indicate that the purified SNV N protein is of sufficient quality and purity to substitute for authentic viral protein in filter binding assays.

TABLE 2

Soluble Purification of the SNV N Protein

Step	Volume (ml)	Total protein (mg)	Estimated purity (%)
Culture medium	3200	ND	ND
NTA	20	3.4	≈70%
SP Sepharose	3	1.8	>85%

Note. ND, not determined.

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